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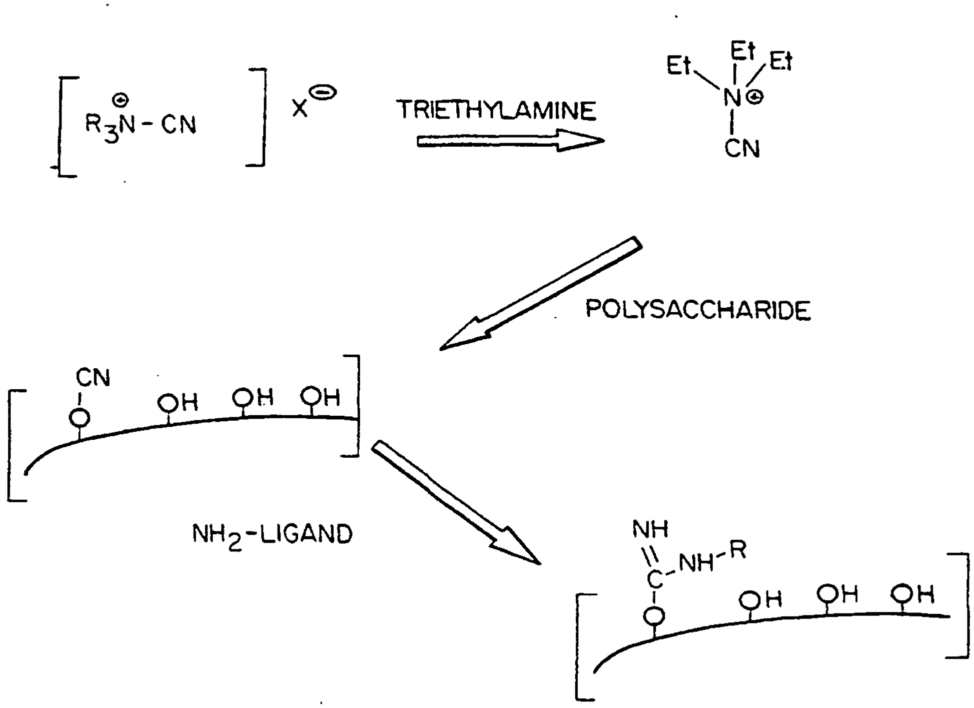

(57) Abstract

A process for producing an immunogenic construct comprising activating at least one first carbohydrate-containing moiety with a novel cyanylating reagent and covalently joining said activated first moiety to a second moiety. Immunogenic constructs may be prepared by this process using either direct conjugation of first and second moieties or using indirect conjugation with a bifunctional reagent.

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METHOD OF ACTIVATING SOLUBLE CARBOHYDRATE USING NOVEL CYANYLATING REAGENTS FOR THE PRODUCTION OF IMMUNOGENIC CONSTRUCTS

U.S. GOVERNMENT INTEREST

The invention described herein may be manufactured, licensed, and used for U.S. governmental purposes without the payment of any royalties to us thereon.

FIELD OF THE INVENTION

This invention relates to improved methods for making immunogenic constructs.

BACKGROUND OF THE INVENTION

In the process of vaccination, medical science uses the body's innate ability to protect itself against invading agents by immunizing the body with antigens that will not cause the disease but will stimulate the formation of antibodies that will protect against the disease. For example, dead organisms are injected to protect against bacterial diseases such as typhoid fever and whooping cough, toxins are injected to protect against tetanus and botulism, and attenuated organisms are injected to protect against viral diseases such as poliomyelitis and measles.

It is not always possible, however, to stimulate antibody formation merely by injecting the foreign agent. The vaccine preparation must be immunogenic, that is, it must be able to induce an immune response. The immune response is a complex series of reactions that can generally be described as follows:

1. The antigen enters the body and encounters antigen-presenting cells which process the antigen and retain fragments of the antigen on their surfaces;

2. The antigen fragments retained on the antigen-presenting cells are recognized by T cells that provide help to B cells; and

3. The B cells are stimulated to proliferate and divide into antibody forming cells that secrete antibody against the antigen.

Certain agents such as tetanus toxoid can innately trigger the immune response, and may be administered in vaccines without modification. Other important agents are not immunogenic, however, and must be converted into immunogenic molecules before they can induce the immune response.

One method of producing immunogenic molecules is provided in related U.S. Patent Application Serial No. 07/834,067, filed February 11, 1992, and its continuation-in-part, Serial No. 08/055,163, filed February 10, 1993, the specifications of which are hereby incorporated. These two related applications describe the dual carrier immunogenic construct, a highly desirable immunogenic construct.

In preparing immunogenic molecules such as the dual carrier-immunogenic construct of U.S. Application Serial Nos. 07/834,067 and 08/055,163 and all other immunogenic constructs, the method used should be sufficiently gentle to retain important antigenic sites, i.e., epitopes on the molecules. Thus, it is desirable to maintain the integrity of the structure and to preserve epitopes in these compounds. Unfortunately, the preparation steps currently used in the prior art are frequently not gentle and can destroy native carbohydrate and/or protein structure. Moreover, most of these techniques for carbohydrate modification require anhydrous conditions but, unfortunately, carbohydrates are

frequently insoluble in organic solvents. Marburg et al., J. Amer. Chem. Soc., 108:5282 (1986).

There are two general methods for producing immunogenic constructs:

(1) direct conjugation of carbohydrate and protein; or

(2) conjugation of carbohydrates and protein via a bifunctional linker or spacer reagent.

Generally, both types of conjugation require chemical activation of the carbohydrate moiety prior to its derivatization. Chemical activation refers to the conversion of a functional group to a form which can undergo additional chemical reactions, e.g., the addition of a functional group or addition of a large moiety such as a protein. Derivatization is the addition of functional chemical group(s) or spacer reagent(s) to a protein.

Certain carbohydrates contain groups, such as amino or carboxyl groups, that can be more easily activated or derivatized before conjugation. For instance, the amino groups in *Pseudomonas* Fisher Type I can be easily derivatized with iodoacetyl groups and bound to a thiolated protein. The carboxyl groups in carbohydrates such as *Pneumonococcal* Type III can be easily activated with water-soluble carbodiimides, such as EDC, and can then be coupled directly to protein. Unfortunately, however, this group of carbohydrates is limited.

Other carbohydrates have aldehyde groups at the terminal reducing end that can be exploited for derivatization and conjugation. It is also possible to create aldehyde groups at the terminal reducing end by treatment with sodium periodate. The presence of aldehyde groups may be beneficial because activation of carbohydrates may not be necessary if aldehyde groups are used.

These aldehyde groups can be condensed with amino groups on protein or with a bifunctional linker reagent. This condensation reaction, especially with the terminal reducing end, however, often proceeds quite slowly and inefficiently.

This is exacerbated when directly conjugating carbohydrate aldehydes to proteins. Thus, yields are often very low using this method. Moreover, sodium periodate may break up carbohydrates into smaller fragments and/or disrupt epitopes, which may be undesirable.

Most carbohydrates, however, must be activated before conjugation, and cyanogen bromide is frequently the activating agent of choice. See, e.g., Chu et al., Inf. & Imm., 40:245 (1983). In brief, cyanogen bromide is reacted with the carbohydrate at a high pH, typically pH 10 to 12. At this high pH, cyanate esters are formed with the hydroxyl groups of the carbohydrate. These, in turn, are reacted with a bifunctional reagent, commonly a diamine or a dihydrazide. These derivatized carbohydrates may then be conjugated via the bifunctional group. The cyanate esters may also be directly reacted to protein.

The high pH is necessary to ionize the hydroxyl group because the reaction requires the nucleophilic attack of the hydroxyl ion on the cyanate ion (CN^-). As a result, cyanogen bromide produces many side reactions, some of which add charged groups and neo-antigens to the polysaccharides. M. Wilcheck et al., Affinity Chromatography. Meth. Enzymol., 104C:3-55. More importantly, however, many carbohydrates can be hydrolyzed or damaged by the high pH which is necessary to perform the cyanogen bromide activation.

In addition, the cyanate ester formed after activation with cyanogen bromide is unstable at high pH and rapidly hydrolyzes, reducing the yield of derivatized carbohydrate and, hence, the overall yield of carbohydrate conjugated to protein. Many other nonproductive side reactions, such as those producing carbamates and linear imidocarbonates, are promoted by the high pH. Kohn et al., Anal. Biochem, 115:375 (1981). Moreover, cyanogen bromide itself is highly unstable and spontaneously hydrolyzes at high pH, further reducing the overall yield.

Furthermore, the cyanogen bromide activation is difficult to perform and unreliable. Cyanogen bromide is highly toxic and potentially explosive. All operations must be carried out in a suitable fumehood. It is also well known to those in the art that the activation is not easily reproducible because some batches of cyanogen bromide work well and some do not. Cyanogen bromide is also poorly soluble in water, making it difficult to control the amount of soluble cyanogen bromide available to react with the carbohydrate. Even use of the same batch of cyanogen bromide and apparently identical reaction conditions do not always lead to identical results.

In addition to these disadvantages, it is very difficult to control the degree of carbohydrate activation achieved by using cyanogen bromide. It is also very difficult to achieve a high level of carbohydrate activation using this method. Increasing the amount of cyanogen bromide present is ineffective and only leads to increased side reactions without an increase in activation. Kohn et al., Applied Biochem and Biotech, 9:285 (1984). Thus, there is a need in the art for a method to produce immunogenic constructs which is gentle, maintains the integrity of the structure of the carbohydrates and proteins, preserves epitopes in the compounds, is easy to perform, is reliable, and is easily reproducible.

SUMMARY OF THE INVENTION

The present invention overcomes the problems and disadvantages of the prior art methods for producing immunogenic constructs by providing a conjugation method that employs a carbohydrate activation method that is safe, easy, inexpensive, and gentle to carbohydrates.

The method of the present invention utilizes novel cyanylating reagents to activate carbohydrate-containing antigens. Because the cyanylating reagent reaction conditions are so gentle, the risk of destruction of

carbohydrate structure and, hence, destruction of naturally-occurring epitopes, is greatly diminished. This method is applicable to a wide variety of soluble carbohydrates and the carbohydrates activated using the method of the invention can be either directly conjugated to protein or can be indirectly conjugated to protein through the use of a spacer or a linker. This method will enable others to produce more effective immunogenic constructs more efficiently and less expensively than immunogenic constructs prepared using prior art methods. As set forth in Table 1 below, this method is more advantageous than the presently used cyanogen bromide.

TABLE 1

Comparison Of Carbohydrate Activation As Preparation For Synthesis Of Conjugates

Cyanogen Bromide	Novel Cyanylating Reagents
High pH (10-12) Destroys many CHO epitopes	Gentle pH (7.0) No alteration of CHO epitopes
Toxic (fume hood required)	Non-toxic
Dangerous in large quantities	Safe
Low yield	High yield
Multiple side reactions	Minimal to no side reactions
Does not easily permit direct conjugation to protein	Allows direct conjugation to protein and enables recovery of unconjugated protein
Batch-to-batch variation	Reproducible
Difficult to work with small quantities	Easy to work with small amounts

In a preferred embodiment, the method of the invention comprises activating a first carbohydrate-containing moiety using the reagent 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP). In another preferred embodiment, the method further comprises directly conjugating the

activated carbohydrate-containing moiety to a second moiety. In another preferred embodiment, the method of the invention comprises activating the carbohydrate-containing moiety using CDAP, covalently binding a bifunctional reagent to the activated moiety and, finally, further reacting the bifunctional reagent with the second moiety, typically a T-dependent antigen, forming a conjugate immunogenic construct, wherein the carbohydrate-containing and TD moieties are linked by a bifunctional reagent.

Additional advantages to using CDAP are (1) the reagent can be made up in advance and stored in a solution for several months; and (2) the concentration of active reagent can be easily determined from its absorbance at 301 nm (Kohn et al., Anal. Biochem, 115:375 (1981)). This makes it possible to standardize the reagent concentration and makes the carbohydrate derivatization more reproducible, which is important for its use in vaccine preparation.

All of the above-mentioned advantages apply both to the direct conjugation of proteins to the carbohydrate and to indirect conjugation via a spacer.

Additional objects and advantages of the invention will be set forth in part in the description which follows and, in part, will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts a generalized scheme for the activation of carbohydrate using cyanylating reagents.

Figure 2 shows schemes for direct conjugation of an activated carbohydrate to protein (left side of figure) and for indirect conjugation of an activated carbohydrate to a protein using a bifunctional reagent (right side of figure).

Figure 3 illustrates a model of an immunogenic construct.

Figure 4 illustrates the incorporation of NH_2 groups into dextran versus the moles of CDAP added/mole of dextran.

Figure 5 illustrates the elution profile of a ^3H-BSA-dextran conjugate from a S400SF gel filtration column.

Figure 6 illustrates the OD280 absorbance of immunogenic constructs prepared according to the method of the invention, eluted from S400SF gel filtration column. A, PT-PRP; B, P28-PT-Pn14.

Figure 7 illustrates the elution profile of $H\delta^a$/1-(CDAP)-dextran from S400SF gel filtration column.

Figure 8 illustrates OD280 and OD430 values of column samples eluted from S400SF gel filtration column loaded with $H\delta^a/NH_2$-(CDAP)-dextran.

Figure 9 illustrates the immunoreactivity of immunogenic constructs prepared using the methods of the invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings.

The invention relates to a method for the activation of carbohydrate-containing antigens for their use in preparation of immunogenic constructs. The invention further relates to a method for the preparation of immunogenic constructs comprising activation of the carbohydrate-containing moiety with a cyanylating reagent. A generalized scheme for the

activation of carbohydrates using cyanylating reagents is shown in Figure 1. Figure 2 illustrates use of an activated carbohydrate for its direct conjugation to protein or its indirect conjugation via the use of a bifunctional linker reagent.

As used herein, the immunogenic construct refers to an entity that can stimulate the immune response. In a preferred embodiment, it is at least one carbohydrate-containing moiety conjugated to at least one second moiety which is a protein. As used herein, "carbohydrate" means any soluble monosaccharide, disaccharide, oligosaccharides, or polysaccharide. Polysaccharides include, but are not limited to, starches, celluloses, and pectins. In another preferred embodiment, the second moiety is a T-dependent antigen, conjugated thereto, as represented in Figure 3.

As used herein, a moiety is any substance which includes, but is not limited to, substances that can be used to stimulate the immune system either by itself or once coupled. Moieties include carbohydrates, proteins, peptides, other antigens, adjuvant molecules, haptens, or combinations thereof. Haptens refer to small molecules, such as chemicals, dust, and allergens, that by themselves are not able to elicit an antibody response, but can once coupled to a carrier. An antigen is any molecule that, under the right circumstances, can induce the formation of antibodies. These haptens and antigens may derive from but are not limited to bacteria, rickettsiae, fungi, viruses, parasites, drugs, or chemicals. They may include, for example, small molecules such as peptides, oligosaccharides (for example the polyribosyl-ribitol-phosphate of *H. influenzae*), toxins, endotoxin, etc.

The process of synthesizing the construct of the invention allows one to advantageously control the physical and chemical properties of the final product. The properties that may be controlled include modifying the charge on the first and second moieties (an advantage in light of evidence

that cationized proteins may be more immunogenic), varying the size of the construct by varying the size of the carbohydrate-containing moiety, selecting the degree of crosslinking of the construct (to obtain variations of size), selecting the number of copies of the second moiety conjugated to carbohydrate-containing moieties, and targeting to selected cell populations (such as to macrophages to enhance antigen presentation). Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds.), Vol. 10, pp. 48-114 (1989).

The immune response to the construct of the invention may be further enhanced by the addition of immunomodulators and/or cell targeting moieties. These entities include, for example, (1) detoxified lipopolysaccharides or derivatives, (2) muramyl dipeptides, (3) carbohydrates, lipids, and peptides that may interact with cell surface determinants to target the construct to immunologically relevant cells, (4) interleukins, and (5) antibodies.

The carbohydrate-containing moiety may be naturally occurring, a semisynthetic or a totally synthetic large molecular weight molecule. In a preferred embodiment, at least one carbohydrate-containing moiety is a carbohydrate selected from the group consisting of *E. coli* polysaccharides, *S. aureus* polysaccharides, dextran, carboxymethyl cellulose, agarose, *Pneumococcal* polysaccharides, Ficoll, crytococcus neo-formans, haemophilus influenzae PRP, *P. aeroginosa*, *S. pneumoniae*, lipopolysaccharides, and combinations thereof.

In the most preferred embodiment, the carbohydrate-containing moiety is a dextran. As used herein, dextran refers to a polysaccharide composed of a single sugar and may be obtained from any number of sources, including Pharmacia. Ficoll, an example of a semisynthetic polymer, is an inert synthetic non-ionized high molecular weight polymer. An

example of a synthetic polymer is polyvinyl alcohol. All are examples of a carbohydrate-containing moiety.

In a preferred embodiment, the second moiety is albumin, a toxoid, a protein, a peptide, a T cell or B cell adjuvant or any other compound capable of activating and recruiting T cell help. The protein may be selected from a group consisting of but not limited to viral, bacterial, parasitic, animal and fungal proteins. In a more preferred embodiment, the second moiety is bovine serum albumin, tetanus toxoid, pertussis toxoid, diphtheria toxoid, heat shock protein, T cell superantigens, or bacterial outer membrane protein, all of which may be obtained from biochemical or pharmaceutical supply companies or prepared by standard methodology (J.M. Cruse & R.E. Lewis, (eds.), Conjugate Vaccines in Contributions to Microbiology and Immunology, Vol. 10 (1989), specifically incorporated herein by reference). Other proteins would be known to those of ordinary skill in the art of immunology.

The second moieties of the invention are capable of being conjugated to at least one carbohydrate-containing moiety. The second moieties may either contain functional groups that can react with the carbohydrate-containing moiety or the second moiety may be chemically manipulated to be capable of reacting with the carbohydrate-containing moiety discussed above.

Numerous copies of specific secondary moieties as well as a variety of second moieties may be conjugated to the carbohydrate-containing moiety. Coupling of multiple copies of the second moiety to the first moiety significantly augments antibody production to the second moiety.

In another embodiment, tertiary moieties may be further conjugated to one or more of the first and/or second moieties. As set forth in the related applications, such conjugation promotes enhanced antibody responses to the tertiary moiety. Techniques to conjugate various moieties to either the primary or secondary moieties are well known to

those skilled in the art, and include, in part, coupling through available functional groups (such as amino, carboxyl, thio and aldehyde groups). See S.S. Wong, Chemistry of Protein Conjugate and Crosslinking CRC Press (1991), and Brenkeley et al., "Brief Survey of Methods for Preparing Protein Conjugates With Dyes, Haptens and Cross-Linking Agents," Bioconjugate Chemistry, 3:1 (Jan. 1992), specifically incorporated herein by reference.

In the method of the invention, the carbohydrate-containing moiety is activated using a cyanylating reagent. Cyanylating reagents increase the electrophilicity of the cyanate and, when reacted with carbohydrate-containing moieties, cyanylating reagents can transfer a cyano group to the hydroxyl groups of the carbohydrate, thus preparing it for further reaction, i.e., direct or indirect conjugation to protein. The activation reaction can be carried out at neutral pH, which improves the stability and integrity of the polysaccharide.

A variety of cyanylating reagents are known, such as N-cyanotriethylammonium tetrafluoroborate (CTEA), 1-cyano-4-(dimethylamino)-pyridinium tetrafluoroborate (CDAP), p-nitrophenylcyanate (pNPC). Wakselman et al. reported that CDAP is a mild reagent that can be used for modification of protein cysteine groups. J.C.S. Chem. Comm., 1976:21 (1976). Of these reagents, CDAP is the most preferred because it is most stable and can be used without a hood, but CTEA and pNPC are also part of the claimed invention. Other tertiary amine complexes with the cyanate group with a variety of counter ions are within the scope of this invention. Particularly useful are non-nucleophilic counter ions such as tetrafluoroborate.

Kohn et al. have compared CDAP, CTEA, and pNPC as activating agents for agarose, an insoluble polysaccharide resin. Kohn et al., Anal. Biochem, 115:375 (1981). Other researchers have used CDAP to activate other types of

insoluble particles, such as Sepharose and glyceryl-controlled pore glass. A. Carpenter et al., Journal of Chromatography, 573:132-135 (1992). Far from the claimed production of immunogenic constructs, these documents disclose the use of activated insoluble particles to prepare gels for affinity chromatography.

In the only report of using CDAP to activate a soluble polysaccharide prior to conjugation with protein, Andersson et al., International Journal of Cancer, 47:439-444 (1991), Andersson et al. directly conjugated epidermal growth factor (EGF) to low molecular weight 40 kDa dextran activated with cyanate. They used very high dextran to EGF ratios of approximately 50:1 (wt/wt) to produce dextran-EGF conjugates and studied the binding of this conjugate to cultured cells, but did not use the conjugate as an immunogen. In fact, it is known that conjugation of proteins to low molecular weight dextrans are poorly or non-immunogenic. T.E. Wileman, J. Pharm. Pharmocology, 38:264 (1985).

In the preferred method, the activation using the cyanylating reagent is performed at pH 6-8 in non-nucleophilic buffer. The cyanylating reagent activation method can be carried out in pH range 6 to 8 in a variety of suitable buffers known in the art. Examples of suitable non-nucleophilic buffers include, but are not limited to, saline, HEPES, phosphate, water and some organic solvents.

In the preferred embodiment of the invention, CDAP is dissolved in a stock solution at a concentration of 100 mg/ml in dry acetonitrile. CDAP concentrations of 0.1 to 10 mg/ml are suitable for use in the method of the invention. Depending on the nature of the carbohydrate-containing moiety used and the degree of activation desired, different concentrations may be optimal.

In the preferred embodiment, the concentration of the carbohydrate-containing moiety is optimally between 1 and 15 mg/ml. The activation reaction can be performed successfully

with concentrations of carbohydrate-containing moiety up to about 100 mg/ml.

In a preferred embodiment, the CDAP to carbohydrate-containing moiety ratio for direct conjugation of protein is between 1:100 and 1:500 per 100 kDa of the carbohydrate-containing moiety. In another preferred embodiment, the CDAP to carbohydrate-containing moiety ratio for indirect conjugation of protein using a spacer is between 1:10 and 1:500 per 100 kDa of carbohydrate-containing moiety. Depending on the nature of the moieties and the conditions used, different moiety ratios may be optimal.

In one preferred embodiment, a carbohydrate-containing moiety which has been activated using a cyanylating reagent is directly conjugated to the second moiety to produce an immunogenic construct. In another preferred embodiment of the invention, the carbohydrate-containing moiety which has been activated with a cyanylating reagent is covalently linked to a suitable bifunctional reagent. Examples of suitable bifunctional reagents include, but are not limited to, ethylene diamine, 1,6-hexane diamine, adipic dihydrazide, cystamine, and lysine, glutamic acid, thiol hydrazides, thiol amines, thiol hydrazides. See, Wong et al., "Chemistry of Protein Conjugate and Crosslinking," CRC Press (1991). The second moiety is then covalently linked to the bifunctional reagent which has already been covalently linked at its other terminus to the carbohydrate-containing moiety.

In a preferred embodiment, triethylamine (TEA) is used to facilitate the cyanylation reaction by the formation of an intermediate Von Braun complex. TEA can be replaced by other tertiary amines capable of forming a Von Braun complex. J. Von Braun, Chem. Ber., 33:1438 (1900).

For certain direct conjugation reactions, glycine amino ethanol or other amino-containing reagents may be used to quench the reaction.

In another embodiment, the invention relates to vaccines that are made up of an immunogenic construct together with a

pharmaceutically acceptable carrier. Such vaccines will contain an effective therapeutic amount of the immunogenic construct together with a suitable amount of carrier so as to provide the form for proper administration to the patient. These vaccines may comprise alum or other adjuvants.

Pharmaceutically acceptable carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil, and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in E.W. Martin, Remington's Pharmaceutical Sciences, specifically incorporated herein by reference.

The vaccines that may be constructed from the immunogenic constructs of the invention may include, but are not limited to, the vaccines set forth in Chart 1.

Chart 1

Diphtheria vaccine
Pertussis (subunit) vaccine
Tetanus vaccine
H. influenzae, type b (polyribose phosphate)
S. pneumoniae, all serotypes
E. coli, endotoxin or J5 antigen (LPS, Lipid A, and Gentabiose)
E. coli, O polysaccharides (serotype specific)
Klebsiella, polysaccharides (serotype specific)
S. aureus, types 5 and 8 (serotype specific and common protective antigens)
S. epidermidis, serotype polysaccharide I, II, and III (and common protective antigens)
N. meningiditis, serotype specific or protein antigens
Polio vaccine
Mumps, measles, rubella vaccine
Respiratory Syncytial Virus
Rabies
Hepatitis A, B, C, and others
Human immunodeficiency virus I and II (GP120, GP41, GP160, p24, others)
Herpes simplex types 1 and 2

CMV
EBV
Varicella/Zoster
Malaria
Tuberculosis
Candida albicans, other candida
Pneumocystis carinii
Mycoplasma
Influenzae virus A and B
Adenovirus
Group A streptococcus
Group B streptococcus, serotypes, Ia, Ib, II, and III
Pseudomonas aeroginosa (serotype specific)
Rhinovirus
Parainfluenzae, types 1, 2, and 3
Coronaviruses
Salmonella
Shigella
Rotavirus
Enteroviruses
Chlamydia trachomatis and pneumoniae (TWAR)
Glycoproteins
Neo-formans cryptococcus

The invention also relates to the treatment of a patient by administration of an immunostimulatory amount of the vaccine. Patient refers to any subject for whom the treatment may be beneficial and includes mammals, especially humans, horses, cows, dogs, and cats as well as other animals, such as chickens. An immunostimulatory amount refers to that amount of vaccine that is able to stimulate the immune response of the patient for the prevention, amelioration, or treatment of diseases. The vaccine of the invention may be administered by any route, but is preferably administered by intravenous, intramuscular, and subcutaneous injections.

The invention also relates to a method of preparing an immunotherapeutic agent against infections caused by bacteria, viruses, parasites, fungi, or chemicals by immunizing a patient with the vaccine described above so that the donor produces antibodies directed against the vaccine. Antibodies would be isolated or B cells may be obtained to later fuse with myeloma cells to make monoclonal antibodies. The method of making monoclonal antibodies is well known in

the art, Kohler et al., Nature, 256:495 (1975), specifically incorporated herein by reference, and needs no further description here. As used herein, immunotherapeutic agent refers to a composition of antibodies that are directed against specific immunogens for use in passive treatment of patients. A plasma donor is any subject that is injected with a vaccine for the production of antibodies against the immunogens contained in the vaccine.

EXAMPLE 1

Derivatization Of A Model Carbohydrate-Containing Moiety With A Spacer

A. Materials

CDAP, pyridine, hexane diamine, sodium borate, HEPES, and triethylamine were purchased from Aldrich (Milwaukee, Wisconsin). The carbohydrate-containing moiety, T2000 dextran, with an average molecular weight of 2000 kDa, was obtained from Pharmacia (Piscataway, New Jersey).

A stock of CDAP in dry acetonitrile at 100 mg/ml was stored at -20°C and kept on ice when in use. T2000 dextran was made up at 10.5 mg/ml in saline + 0.02% azide. Aqueous triethylamine stock was made up at 0.2 M and kept on ice during use.

Hexane diamine was made up at 0.5 M in 0.1 M sodium borate.

Amino group determination was made using trinitrobenzene sulfonate (TNBS) and an extinction coefficient of 11,000 m^{-1} at 366 nm. Franci et al., J. Imm. Methods., 86:155 (1986). Carbohydrate was assayed by the method of M. Monsigny et al., Anal. Chem., 175:525 (1988) using T2000 dextran as the standard.

B. Control Reactions

The following experiments demonstrate that all components used in the derivatization reaction of the invention are important and that the amino groups in the

final conjugate are covalently linked to the carbohydrate and their presence is not due to artifact or "carryover" of reagent into the final product. Reactions were carried out on ice. For trials performed, omission or substitution of reagents was as indicated in Table 2.

In the procedure using all reagents, line 1 of Table 2, CDAP was added to a vortexed solution of 300 μl dextran (3.1 mg) and returned to the ice bucket. Thirty seconds later, the TEA was added to the vortexed solution. Two minutes after the CDAP was added, 200 μl of the diamine was added and the solution kept on ice for another hour. Samples were dialyzed overnight, filtered with a Millex GV filter and further desalted on a 1 x 15 cm P6DG column (BioRad).

As shown in Table 2 below, incorporation of amino groups into dextran required the presence of dextran, CDAP, TEA, and hexane diamine. The data in Table 2 further demonstrate that the amino groups detected are not due to carryover of unconjugated reagents into the final products. Without TEA (transfer reagent) present, there is only minimal derivatization.

TABLE 2

#	Saline	Dex	100 mg/l CDAP	0.2 M TEA	0.5 M Hexane Diamine	0.1M Borate	NH_2/ Dex
1	0	300μl	15μl	15μl	300μl	0	1274
2	300μl	0	15μl	15μl	300μl	0	0
3	0	300μl	0	15μl	300μl	--	0
4	0	300μl	15μl	0	300μl	--	42
5	0	300μl	15μl	15μl	0	300μl	0
6	300μl	0	15μl	0	0	0	0

C. Derivatization Of T2000 Dextran With Hexane 1,6-Diamine

This experiment demonstrates that CDAP can be used to derivatize carbohydrates to introduce amino groups at both high and low ratios. Dextran T2000 was used as a model carbohydrate. Dextran is a polymer made up of glucose monomers.

The first step in the preparation of many conjugate vaccines is the addition of a spacer (Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds.), Vol. 10, pp. 48-114 (1989)). This series of experiments, summarized in Table 3, emphasizes the ease with which a spacer can be added to polysaccharides.

TABLE 3

#	Dextran (µl)	CDAP (µl)	TEA (µl)	Diamine (µl)	10^{-3} mole CDAP/mole Dextran	NH_2/* Dextran	% Efficiency (NH2/CDAP**	% *** Derivatized
1	600	5	5	600	.68	340	50.0	3.1
2	600	10	10	600	1.36	650	48.5	5.9
3	600	15	15	600	2.03	500	24.8	4.6
4	300	15	15	200	4.06	600	16.7	6.1
5	300	30	30	200	8.12	956	11.8	8.2
6	300	60	60	200	16.24	1684	4.2	6.2
7	300	120	120	200	32.48	2233	6.9	20.4
8	300	15	15	200	4.06	760	18.7	6.9****
9	300	30	30	200	8.12	1240	15.3	11.3****
10	300	60	60	200	16.2	700	4.3	6.4****
11	600	15	15	600	2.03	380	18.8	3.5

* Moles NH_2 per 2000 kDa of dextran.

** To calculate this value, NH_2/dextran values were divided by mole CDAP/mole dextran values and multiplied by 100%.

*** Percent of glucose units within dextran bound to an NH_2 group.

**** Experiment carried out at room temperature.

The experiment was conducted at two temperatures. In lines 1-7 and 11, all reagents were kept on ice and in lines 8-10, they were at room temperature. Procedures and reagents were used as described above for Table 2 and reagent amounts added are indicated in Table 3. In line 11, diamine was added in 0.15 M HEPES. The reaction was slightly less efficient at lower pH. In another embodiment, hexane diamine was made up in 0.1 M borate, pH 9.

Efficiency is defined as the number of moles of spacer groups incorporated per mole of CDAP used, expressed as a percentage. The last column, "% derivatized," is the percent of the glucose monomer units of the dextran which have been modified with a spacer.

The results are further illustrated in Fig. 4, which shows the total number of amino groups (e.g., the spacer reagent added) incorporated versus the moles of CDAP added per moles dextran unit. When this data is converted into NH_2 incorporation versus moles CDAP/mole dextran, it is evident that a CDAP:glucose ratio of less than one is sufficient for high levels of NH_2 incorporation. Thus, minimal modification of dextran polysaccharide is necessary for high NH_2 group incorporation.

Furthermore, since an undetermined amount of the active cyanate ester is hydrolyzed without adding a spacer, the CDAP/glucose ratio is in fact an overestimate of the degree of modification of the polymer. Thus, the actual degree of modification is less than the calculated CDAP/glucose ratio.

The degree of incorporation of spacer groups at the lowest reagent dose tested (line 1), 3.1%, is comparable to that used for the synthesis of conjugate vaccines (Chu et al., Inf. & Imm., 40:245 (1983); Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds.), Vol. 10, pp. 48-114 (1989).

The table and figure demonstrate the high efficiency of the CDAP reaction for adding spacer reagents. Further

optimization of reaction conditions can increase efficiency. Also illustrated is the very high level of incorporation of spacer groups into polysaccharide which is possible using CDAP. At the highest amount of CDAP added (line 7) approximately 1 in 5 of the glucose units were modified (20%) with a spacer. It is not possible to obtain this degree of incorporation of spacer with cyanogen bromide (Kagedal & Akerstrom, Acta Chemica Scan., 25:1855 (1971)).

During the reactions, there was no evident precipitation of the dextran polysaccharide. In contrast, aggregation and precipitation of the polysaccharide can be a problem with the cyanogen bromide method (Kagedal & Akerstrom, Acta Chemica Scan., 25:1855 (1971)).

These reactions were done in small volumes (<1 ml), thus allowing many trial experiments to be conveniently performed. This is important when optimizing a procedure without wasting valuable carbohydrates. In contrast, it is difficult to conveniently work with very small amounts of cyanogen bromide due to its poor water solubility, uncertain potency, and toxicity.

Moreover, comparing lines 8-10 of Table 3 with lines 1-7 and 11, it appears that the level of incorporation of amino groups into dextran was approximately the same when the coupling reaction was carried out at 0°C or room temperature.

D. Demonstration Of Efficiency Of Conjugation Reaction Using CDAP And Verification Of Conjugation Using Radiolabeled Protein

Since the conjugation reaction using CDAP caused some absorbance at 280 nm, the wavelength normally used to estimate protein concentrations, radiolabeled protein was directly conjugated to dextran. This allowed independent determination of the protein concentration from its specific activity. The yields and recovery of protein were determined.

1. BSA was lightly radiolabeled with N-hydroxysuccinimide (^{3}H-2,3)propionate (Amersham), essentially as described by Brunswick et al. Radiolabeled BSA was dialyzed exhaustively into PBS + 0.02% azide and subjected to gel filtration chromatography on a S100HR column (Pharmacia) to remove aggregates and concentrated by ultrafiltration using a YM30 filter (Amicon). The BSA concentration was 21 mg/ml, determined from its extinction coefficient at 280 nm (44,000 M^{-1}). The specific activity of the stock solution, determined by liquid scintillation counting, was 5.48×10^{12} cpm/mole.

2. Other reagents were as follows: T2000 dextran (approximately 2000 kDa) (Pharmacia) was dissolved at 10.5 mg/ml in water. CDAP was made up at 100 mg/ml in dry acetonitrile, triethanolamine (TEA) was made up at 0.2 M in water. Glycine (pH 5.0) was prepared at 1 M in water.

3. Protocol: Reagents were kept on ice and all reactions were performed on ice. The reaction mixture was vortexed during each addition. 25 µl of CDAP was added to 0.5 ml of dextran (5.25 mg) and 30 seconds later 25 µl TEA was added. After a total of 2.5 minutes, 5.25 mg of radioactive BSA was added. 30 minutes later, the reaction was quenched by the addition of 100 µl of glycine solution and left overnight at 4°C. An aliquot of 0.6 ml was then filtered using a Spin-X membrane (COSTAR). A comparison of the radioactivity aliquots before and after filtration demonstrated that essentially 100% of the radioactivity was recovered in the filtrate.

500 µl of the filtrate was applied to a 1 x 57 cm S400SF gel filtration column (Pharmacia) which was equilibrated with saline plus 0.02% azide, and run at 0.2 ml/min. Fractions of 0.89 ml were collected and analyzed. Dextran concentrations were determined by the method of Monsigny et al. using absorbance at 480 nm. The radioactivity of 50 µl aliquots taken from each tube were determined by liquid scintillation counting and (^{3}H)BSA

concentrations were calculated using its specific activity. The position of unconjugated BSA in the column elution was determined in an independent column run.

4. As shown in Figure 5, a large portion of the BSA, represented by the cpm, is in a high molecular weight form which runs in an identical position as the dextran, represented by OD480. There is a small residual BSA peak representing unconjugated protein. Table 4 contains the purification data.

TABLE 4

Total protein recovered	:	3.0 mg
Protein applied to column	:	2.9 mg
Recovery	:	103%
Protein in high MW form (tubes 15-23)	:	>2.0 mg (68%)

Ratio of BSA : DEXTRAN for 2000 kDa dextran : 26

The column did not cleanly separate the dextran-BSA conjugate from the unconjugated protein. This is not unusual since the high molecular weight polymers frequently cause tailing in gel filtration columns. Furthermore, since the T2000 dextran was unfractionated, it contained a spectrum of sizes. To estimate the amount of conjugated BSA in the region where free and bound BSA overlap, we assumed a constant ratio of bound BSA to dextran. Total conjugated BSA, calculated by multiplying the BSA:dextran ratio x the total molar amount of dextran, was determined as 2.55 mg. This indicates that 87% of the protein was converted to conjugate form.

TABLE 5

Mole CDAP/ mole glucose	mole TEA/ mole CDAP	BSA/dextran	% BSA Conjugated
0.39	1:2	26	88
0.39	2:1	10	34
0.16	1:2	9	28
0.16	5:1	1	3

The results of this BSA-dextran experiment are summarized in Table 5 (line 1) along with three other trials using different amounts of CDAP and TEA (lines 2-4). Both the amount of TEA and the amount of CDAP are critical in order to get high protein to polysaccharide ratios via direct conjugation. The optimal reagent quantities can easily be determined since the method permits convenient experimentation with small amounts.

It should be emphasized that the direct conjugation reaction does not modify the unconjugated protein, unlike the carbodiimide or heteroligation coupling methods, nor does it use harsh conditions. Thus, one could recover the unconjugated protein for further use. Since many protein antigens are valuable, this is a major advantage of the direct conjugation method.

EXAMPLE 2

Preparation Of PT-Pn14 Conjugates

The purpose of these experiments is to (1) demonstrate that the transformation of the protein from a low molecular weight form to a high molecular weight form was a result of direct conjugation of the protein to the carbohydrate and (2) determine, under one particular set of conditions, the minimum amount of cyanylating reagent needed to conjugate the protein.

Pertussis toxoid (PT) (from Mass. Public Health Biol. Labs, Boston, MA) was dissolved at 0.289 mg/ml in 0.5 M NaCl, 0.02 M NaPhosphate, pH 8.8. 0.1 ml of 0.1 M sodium borate pH

9.1 or 0.75 M HEPES, pH 7.5, was added per milliliter of PT. *Pneumococcal*-type 14 (Pn14) (ATTC lot 83909) was dissolved at 5 mg/ml in 0.15 M saline with 0.02% azide. Triethylamine (TEA) was dissolved at 0.2 M in water. CDAP was dissolved at 100 mg/ml or 10 mg/ml in acetonitrile (made up and stored at -20°C). Glycine was made up at 1.0 M, pH 5.0. Amino ethanol or other amino reagents can be substituted for glycine/HCl.

Experiment 1 - **Conjugation of Pertussis toxoid to Pn14**

Each tube contained 250 μg of Pn14 (50 μl) on ice. At time zero, various amounts of CDAP as indicated in the table were added and 30 seconds later 25 μl of TEA was added. Two minutes later 1 ml of PT was added. After about 1 hour, 100 μl of glycine solution was added.

Samples were kept at 4°C overnight. The next day, they were filtered with a Costar 0.45 micron spin filter and run on an HPLC TSK-gel filtration column in 0.2 M KCl. % HMW is the area of the high molecular weight OD280 conjugate peak versus the OD280 peak indicating unconjugated moiety. It is defined by (percent area void volume peak/(% area void vol. peak + % area unconjugated moiety peak). The percent areas, obtained from the HPLC runs, were as follows:

TABLE 6

Direct Conjugation Of Pertussis Toxoid To Pn14

#	μmole CDAP/100 kDa Pn14	% HMW
1	1720	100.0
2	520	52.3
3	172	32.8
4	51	31.0
5	17	28.1
6	0 (PT control)	22.0
7	0; no TEA, no PT, (Pn14 control)	--
8	0; no TEA, no Pn14; PT without Borate	11.3

Because the PT control has a % HMW of 22%, there may be a small amount of aggregation of the PT caused by the

reaction conditions. This set of data also indicates that by varying the CDAP to protein ratio, it is possible to control the ratio of protein to carbohydrate in the final conjugate.

Experiment 2 - Conjugation Of A Monosaccharide To PT

In this series, 150 µl of a solution of 10 mg/ml glucose, which is monomeric, was substituted for the Pn14 polysaccharide. Conditions similar to Experiment 1 were used except that the PT was made up in HEPES (pH 7.5, M 0.075) buffer instead of borate. Also, 20 µl instead of 25 µl TEA was used. These conditions yielded the following:

#	Condition	% HMW form
1	PT only, no CDAP or TEA	<20%
2	CDAP, TEA (no glucose); + PT	~0
3	Glucose, CDAP, TEA; + PT	~0

Numbers 2 and 3 indicate that CDAP does not polymerize the pertussis toxoid itself and that, therefore, the conversion of the PT to a high molecular weight form is due to its coupling to the high molecular weight polysaccharide and not due to polymerization of the protein. It was evident from the HPLC run that glucose was conjugated to PT because there was a slight increase in the molecular weight of PT.

Experiment 3 - Synthesis Of Useful Vaccine Construct With A Spacer: Pertussis Toxoid-Pn14

Pn14-derivatized with hexane diamine was prepared as follows. 10 µl of CDAP (100 mg/ml in acetonitrile) was added (193 mole CDAP per 100 kDa of polysaccharide). Thirty seconds later 20 µl of TEA (0.2 M) was added. After a total of 2.5 minutes had elapsed, 300 µl of 0.5 M hexane diamine in 0.1 M sodium borate (pH 9.1) was added. After one hour, the solution was dialyzed into water, filtered, and desalted

into saline on a P6DG (BioRad) column. The void volume was pooled and concentrated with a Centricon 30 device (Amicon). It was determined to have 33 amino groups per 100 kDa of Pn14 polysaccharide.

Pertussis toxoid was conjugated to the amino-Pn14 using heteroligation chemistry (Brunswick et al.). 50 μl of 0.75 M HEPES buffer (pH 7.5) was added to 0.44 ml of the amino-Pn14. It was iodoacetylated with 10 μl of 0.1 M iodoacetyl propionate N-hydroxy-succinimide (SIAP). Pertussis toxoid was thiolated with a 20-fold molar excess of SATA (Calbiochem, La Jolla, CA). Each was desalted into saline, mixed, and 1/9 volume of buffer containing 0.75 M HEPES, 10 mM EDTA, and 0.5 M hydroxylamine was added. The final volume was 1.1 ml. After an overnight incubation, the solution was made 0.2 mM in mercaptoethanol for one hour and then 10 mM in iodoacetamide for 10 minutes, following which it was fractionated on a S400SF gel filtration column (Pharmacia) (see Fig. 6). The void volume peak was pooled and concentrated by pressure filtration on a PM10 membrane (Amicon). Approximately 50% of the pertussis toxoid was recovered in conjugate form. The final conjugate contained 0.7 moles PT per 100 kDa of Pn14 polysaccharide. Protein concentration in the conjugate were determined by the Bradford assay (BioRad) using PT as the standard. Polysaccharide concentrations were determined by the method of Monsigny et al. using Pn14 as the standard.

CTEA offers the advantage of having fewer side reactions than CDAP and leads to purer products, as described in Kohn et al., <u>Anal. Biochem</u>, 115:375 (1981). Its disadvantage is that it is moisture sensitive, must be weighed out in a closed vessel, and cannot easily be prepared as a stock solution.

Direct Conjugation Of A Protein To Pn14 Using CTEA

1 ml of *Pneumococcal*-type 14 polysaccharide (Pn14) (5 mg/ml in saline) is kept at 0°C. CTEA (Available from Aldrich Chemical, Milwaukee, WI) is stored under dry nitrogen. 2 mg CTEA is weighed out in a closed weighing vessel and added to the cooled, vigorously mixed Pn14. 20 μl of TEA (0.2 M in water) is immediately added while mixing. Sixty seconds later, 5 mg of pertussis toxoid (1.5 mg/ml) is added to the stirred solution. One-half hour later, the reaction is quenched with 200 μl 1 M glycine (pH 5.0). After an additional hour, the solution is filtered and passed over an S400SF gel filtration column, equilibrated with saline. The void volume peak is collected and sterile filtered. A 1:1 conjugate is produced.

Addition Of Spacer Reagent To *Pneumococcal* Type 14 Polysaccharide Using CTEA

1 ml of Pn14 (5 mg/ml in saline) is kept at 0°C. CTEA (available from Aldrich Chemical, Milwaukee, WI) is stored under dry nitrogen. 1 mg CTEA is weighed out in a closed weighing vessel and added to the cooled, vigorously mixed Pn14. Immediately add 20 μl to TEA (0.2 M in water) while mixing. Sixty seconds later, 300 μl of 0.5 M hexane diamine in 0.1 M borate (pH 9) is added while mixing. After one hour, the solution is exhaustively dialyzed into saline and sterile filtered. Since a ratio of 187 mole CTEA per 100 kDa Pn14 is used, a conjugate with approximately 18 amines per 100 kDa of Pn14 is produced.

EXAMPLE 3

Direct Conjugation Of Pertussis Toxoid To Haemophilus Influenzae Polysaccharide (PRP)

PRP, average MW 350 kDa, was obtained from the Massachusetts Public Health Biological Laboratory. Pertussis

toxoid was from the same source. 15 µl of CDAP (100 mg/ml) was added to 100 µl (2 mg) of PRP on ice. Thirty seconds later, 30 µl of TEA was added. This represented 319 moles of CDAP per 100 kDa of PRP. After an additional two minutes, 0.75 ml of pertussis toxoid (1.1 mg) was added. Forty minutes later, 200 µl of 1 M glycine (pH 5.0) was added to quench the reaction. After one additional hour, the solution was passed over an S400SF gel filtration column equilibrated with saline (see Fig. 7). The void volume was pooled and sterile filtered. The product was determined to have 1.1 PT per 100 kDa of PRP with an overall yield of 68%.

The vaccine prepared by Chu et al., Inf. & Imm., 40:245 (1983) used 377 mole cyanogen bromide per 100 kDa of PRP and had ratios of 1.4 to 2.1 PT per 100 kDa of PRP with yields of less than 50%. Thus, the direct conjugation method of the invention yielded a similar conjugate but with less work, higher yields and without the use of a toxic reagent.

Since many published protocols for preparing PRP conjugates start with the PRP derivatized with a spacer (Chu et al., Schneerson et al., J. Exp. Med., 152:361 (1980); Dick & Beurret, "Glycoconjugates of Bacterial Carbohydrate Antigens," Conjugate Vaccines, J.M. Cruse & R.E. Lewis (eds.), Vol. 10, pp. 48-114 (1989), CDAP was also used to add a spacer to PRP.

The conditions used were as described above but 100 µl of 0.1 M hexane diamine in 0.1 M borate was added instead of the pertussis toxoid. The product was dialyzed into saline. It was determined to have 102 amino groups per 100 kDa of PRP. Since this is a higher ratio than used in published procedures, even less CDAP could have been used.

EXAMPLE 4

Immunogenic Constructs Useful As Vaccines Prepared Using CDAP Chemistry

A. Conjugation Using CDAP And A Bifunctional Reagent

In brief, malaria-derived peptide P28, CNIGKPNVQDDQNK, from the gamete-specific protein pfs25, was conjugated to tetanus toxoid (TT). P28 has been shown to induce malaria transmission blocking antibodies. CDAP was then used to couple p28-TT to *Pneumococcal*-type 14 (Pn14) polysaccharide.

FDA-approved tetanus toxoid was dialyzed overnight into HEPES buffer and reacted with a 30-fold molar excess of the iodoacetylating agent (SIAP). After 3 hours, reagents were removed by ultrafiltration using a Macrosep 30 (Filtron Technology) and washed into fresh HEPES, 0.15 M, pH 7.5 buffer. Tritium labeled P28 was added as a solid to the derivatized TT while gently mixing. Following overnight reaction at 4°C, the mixture was treated with 0.2 mM mercaptoethanol to block any remaining active groups and then desalted on a P6DG column equilibrated with HEPES buffer. From the specific activity of the peptide, the product was determined to contain 20 moles P28 peptides/mole of TT. The conjugate was dialyzed into saline and sterile filtered.

B. Direct Conjugation Using CDAP

Pn14 (obtained from American Tissue Type Collection) has a high molecular weight (c.a. 10^6 daltons). P28-TT was directly conjugated to Pn14 as follows. CDAP (10 μl from a 100 mg/ml stock solution in acetonitrile) was added to Pn14 (1.1 mg in 150 μl saline). 30 seconds later, 20 μl of triethylamine (0.2 M) was added. Two minutes later, 0.55 mg (in 0.8 ml saline) of P28-TT was added and one hour later, the reaction was quenched for another hour with 200 μl 1.0 M glycine (pH 5). The conjugate was then passed over an S400SF gel filtration column equilibrated with saline and the void volume containing the conjugate was pooled.

Figure 9 indicates that virtually all of the P28-TT was found in the void volume in conjugated form.

C. Immunoreactivity Of Immunogenic Constructs

Groups of 5 DBA/2 mice were immunized with i.v. with 10 μg P28-TT or (P28-TT)-Pn14 conjugate, in saline, bled three weeks later, and the sera assayed by ELISA for reactivity against recombinant pfs25 protein. Peptide P28 is derived from pfs25. Another set of mice was immunized with the same antigens precipitated with the adjuvant, alum (Imject, Pierce Chemical Co., Rockford, IL).

Consistent with the related applications, Table 7 shows that only the high molecular weight conjugate elicited good anti-protein titers.

TABLE 7

Anti-pfs25 IgG1 Titers

Antigen	i.v. (saline)	s.c. (alum)
(P28-TT)-Pn14	36	346
P28-TT	<10	<10

This demonstrates that the CDAP method can be used to prepare useful vaccine constructs. It also illustrates the ease with which useful conjugates can be prepared.

EXAMPLE 5

Biologically Active Multivalent Protein Constructs Prepared Using CDAP

To demonstrate that conjugates prepared using CDAP to directly couple proteins to polysaccharides could yield a multivalent product (which as set forth in the related applications has enhanced immunogenicity) and that the process could be gentle enough to preserve biological activity, various conjugates of a monoclonal antibody with dextran were prepared. These experiments used monoclonal

antibody $H\delta^a/1$ with an anti-IgD antibody which crosslinks membrane IgD on B lymphocytes and induces proliferation (Brunswick et al., Journal of Immunol., 140:3364 (1988)). As described by Brunswick et al., conjugation of multiple copies of $H\delta^a/1$ to a high molecular weight polymer such as 2000 kDa dextran ($H\delta^a/1$-AECM dextran) induced B cell proliferation at 1000-fold lower concentrations and induced higher levels of proliferation than unconjugated $H\delta^a/1$. In Brunswick et al., a simple, straightforward but multistep, multi-day procedure was required to prepare the conjugate. Aminoethyl carboxymethyl dextran (AECM dextran) was prepared first as described in Brunswick et al. and then heteroligation chemistry was used to couple the $H\delta^a/1$ to the carbohydrate.

$H\delta^a/1$-dextran was prepared by both direct conjugation using CDAP and indirect conjugation using a spacer and CDAP as follows.

Direct conjugation: To a vortexed solution of 3.2 mg of T2000 dextran (Pharmacia) in 0.3 ml saline, 15 μl of CDAP was added (from a 100 mg/ml stock in acetonitrile). 30 seconds later, 15 μl of 0.2 M TEA was added while vortexing. After an additional 2 minutes, 6 mg $H\delta^a/1$ (in 362 μl 0.05 M sodium borate and 0.075 M NaCl) was added while gently vortexing. After 15 minutes, the reaction mixture was quenched by the addition of 100 μl of 1.0 M glycine, pH 5.0 and passed over an S400SF gel filtration column (1 x 59 cm) equilibrated with saline. The column elution is shown in Figure 9. The void volume peak was pooled and sterilized with a Millex GV filter. The product is called $H\delta^a/1$-(CDAP)-dextran. This procedure took approximately 3 hours.

Spacer: Dextran was activated with CDAP as above (31.5 mg T2000 dextran in 3 ml saline and 25 μl CDAP followed by 25 μl TEA, 1 mole CDAP/0.06 mole of glucose monomers). 3 ml of 0.5 M 1,6-diaminohexane in 0.1 M sodium borate was added. The solution was exhaustively dialyzed into water and then fractionated on an S400HR gel filtration column. The void volume was pooled and concentrated. This amino-dextran was

determined to have 147 amino groups per 2000 kDa dextran. The product is called NH_2-(CDAP)-dextran. Including dialysis, this was a two-day procedure. In contrast, AECM-dextran usually takes about one week to prepare using the Brunswick et al. method.

$H\delta^a/1$ was conjugated to AECM-dextran and NH_2-(CDAP)-dextran using the heteroligation techniques described in Brunswick et al. The conjugates are called $H\delta^a/1$-AECM-dextran and $H\delta^a/1$-NH_2-(CDAP)-dextran, respectively. Conjugation using ACEM-dextran was a two-day procedure.

B cell proliferation assays, using 10,000 cells/well, were performed as described by Brunswick et al. Table 8 provides the results of those assays, specifically indicating incorporation of tritiated thymidine into B cells as counts per min/well.

TABLE 8

	$H\delta^a/1$ Concentration (μg/ml)		
Mitogen	1	0.1	0.01
$H\delta^a/1$-AECM-dextran (preparation 1)	16,045	25,774	25,850
$H\delta^a/1$-AECM-dextran (preparation 2)	21,685	29,280	34,969
$H\delta^a/1$-(CDAP)-dextran	16,497	23,654	19,779
$H\delta^a/1$-NH_2-(CDAP)-dextran	19,353	28,343	25,879
Medium (control)	760	725	760

Not shown: As reported in Brunswick et al., $H\delta^a/1$ alone causes no incorporation at these concentrations. Maximum incorporation, at 10-100 μg/ml $H\delta^a/1$, is approximately 3000 cpm.

These data indicate that the conjugates prepared using CDAP, with and without a spacer, are essentially equivalent to $H\delta^a/1$-AECM dextran in their abilities to induce proliferation. Since only multivalent antibody induces high

levels of proliferation at low doses, all the conjugates must be multivalent. Thus, direct conjugation with CDAP did not affect the biological activity of the antibody. The direct conjugation procedure was markedly faster to prepare than conjugates prepared with a spacer. Further, adding the spacer and conjugating using CDAP was much faster than preparing AECM dextran.

Thus, this experiment illustrates (1) the high yield of a multivalent construct using CDAP and (2) the ease and speed of preparation of conjugates, especially direct conjugates. Conjugation using CDAP and a bifunctional reagent took under 48 hours and direct conjugation took less than three hours.

It will be apparent to those skilled in the art that various modifications and variations can be made in the methods of the present invention for the construction of immunogenic constructs without departing from the scope or spirit of the invention.

Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

WHAT IS CLAIMED IS:

1. A process for producing an immunogenic construct comprising:

a) activating at least one first carbohydrate-containing moiety with a cyanylating reagent; and

b) covalently joining said activated carbohydrate to a second moiety.

2. A process for producing an immunogenic construct comprising:

a) activating at least one first carbohydrate-containing moiety with a cyanylating reagent;

b) covalently joining said first moiety to a bifunctional spacer reagent; and

c) covalently joining a second moiety to the bifunctional spacer reagent of step b).

3. The method of claim 1 or 2, wherein the cyanylating reagent is selected from the group consisting of CDAP, CTEA, and pNPC.

4. The method of claim 1 or 2, wherein the immunogenic construct is a dual carrier construct.

5. The method of claim 1 or 2, wherein said bifunctional reagent is selected from the group consisting of ethylene diamine, 1,6-hexane diamine, adipic dihydrazide, cystamine, glycine, and lysine.

6. A dual carrier immunogenic construct produced according to claim 1 or 2.

7. The method of claim 1 or 2, wherein the first moiety is selected from the group consisting essentially of dextran, *Pneumococcal* polysaccharide, haemophilus influenzae

polysaccharide, a viral polysaccharide, and a bacterial polysaccharide.

8. The method of claim 1 or 2, wherein the second moiety is selected from the group consisting of BSA, pertussis toxoid, tetanus toxoid, malaria-derived peptide P28, an antibody, a toxoid, and a toxin.

9. An immunogenic construct produced according to claim 1 or 2, which is selected form the group consisting essentially of PT-Pn14, PT-PRP20, TT-Pn14, $H\delta^{a}$/1-dextran, and P28-TT-Pn14.

10. A method for producing an immune response, comprising:

a) producing an immunogenic construct by

i) activating at least one first carbohydrate-containing moiety with a cyanylating reagent, and

ii) covalently joining said activated carbohydrate to a second moiety;

b) administering the immunogenic construct to a patient.

11. A method for producing an immune response, comprising:

a) producing an immunogenic construct by

i) activating at least one first carbohydrate-containing moiety with a cyanylating reagent,

ii) covalently joining said first moiety to a bifunctional spacer reagent, and

iii) covalently joining a second moiety to the bifunctional spacer reagent of step ii); and

b) administering the immunogenic construct to a patient.

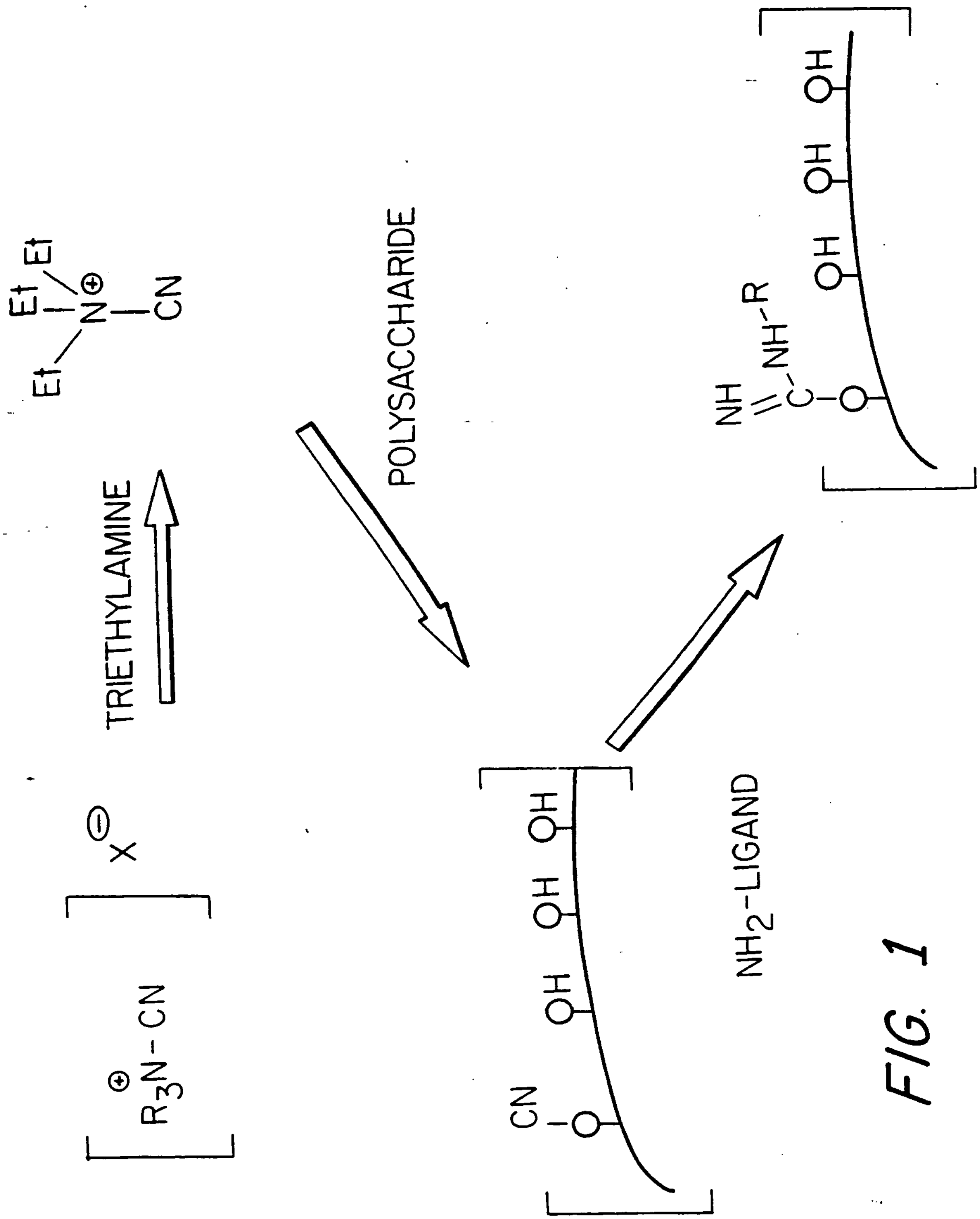

FIG. 1

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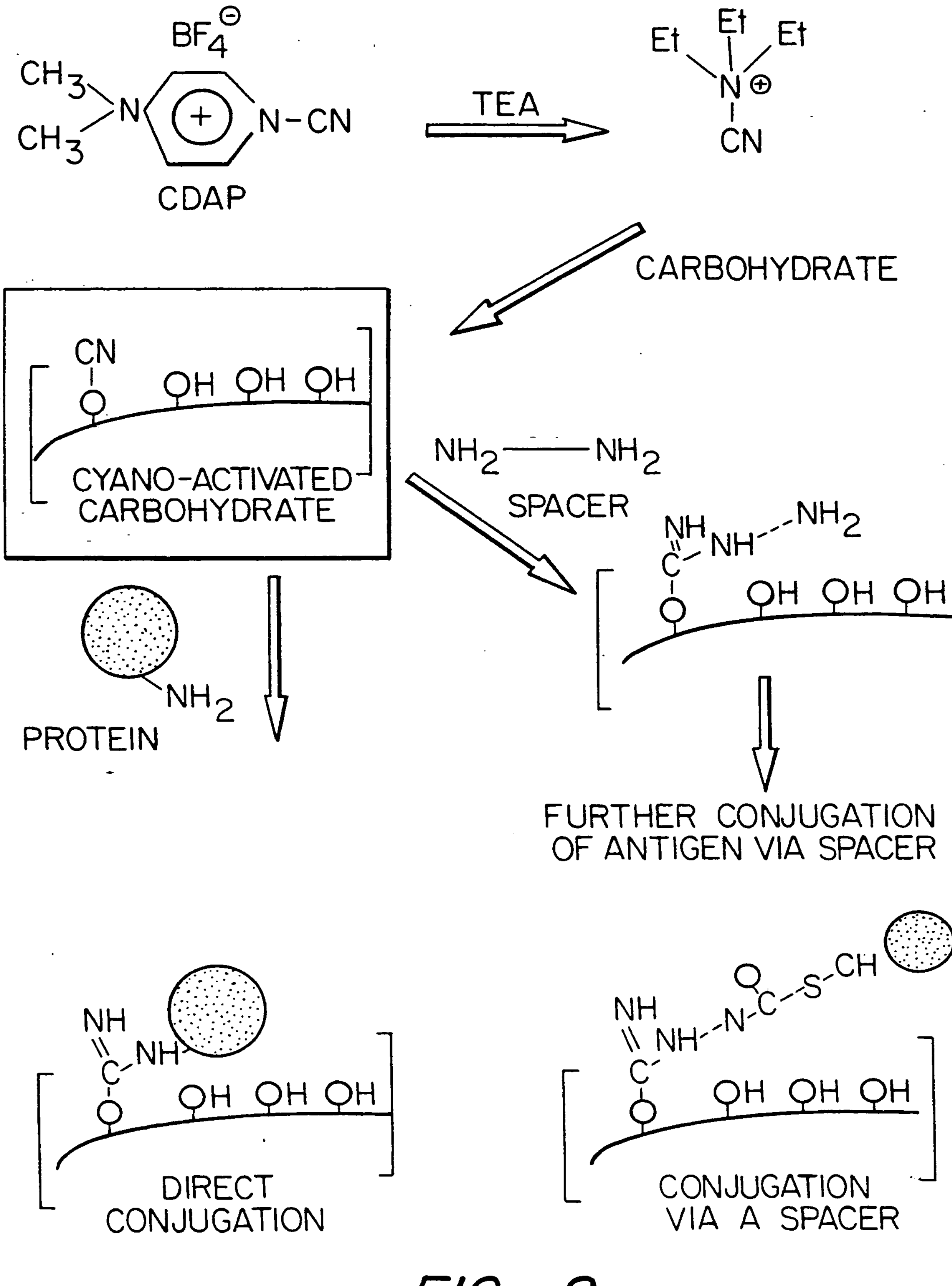
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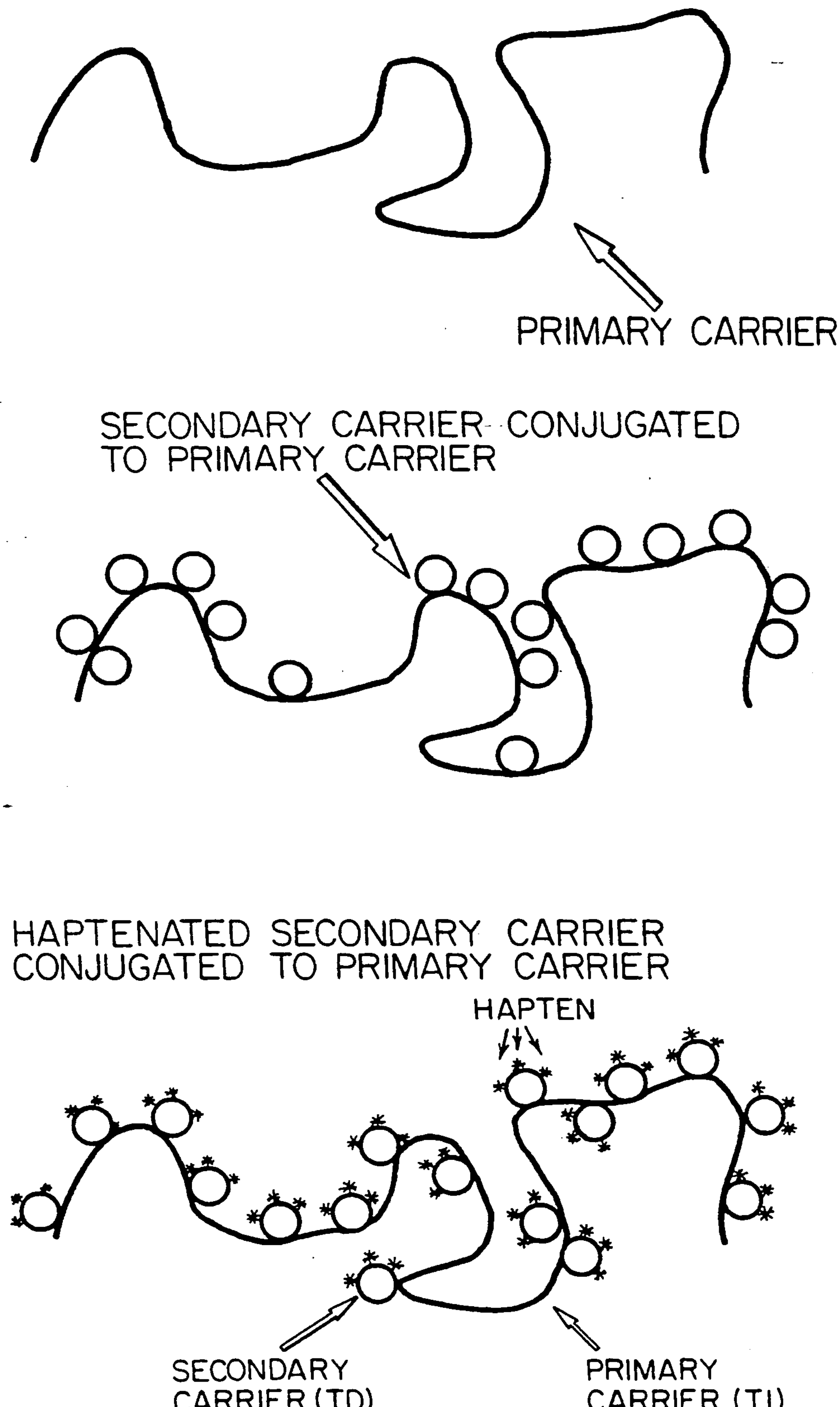
CH3
CH3
N
N-CN
CDAP
TEA
Et
Et
Et
N
CN
CARBOHYDRATE
CN
OH OH OH
CYANO-ACTIVATED
CARBOHYDRATE
NH2 NH2
SPACER
NH
NH
NH2
C
OH OH OH
NH2
PROTEIN
FURTHER CONJUGATION
OF ANTIGEN VIA SPACER
NH
NH
C
OH OH OH
DIRECT
CONJUGATION
NH
NH
N
C
S
CH
C
OH OH OH
CONJUGATION
VIA A SPACER

FIG. 2

FIG. 3


PRIMARY CARRIER
SECONDARY CARRIER CONJUGATED TO PRIMARY CARRIER
HAPTENATED SECONDARY CARRIER CONJUGATED TO PRIMARY CARRIER
HAPTEN
SECONDARY CARRIER (TD)
PRIMARY CARRIER (TI)

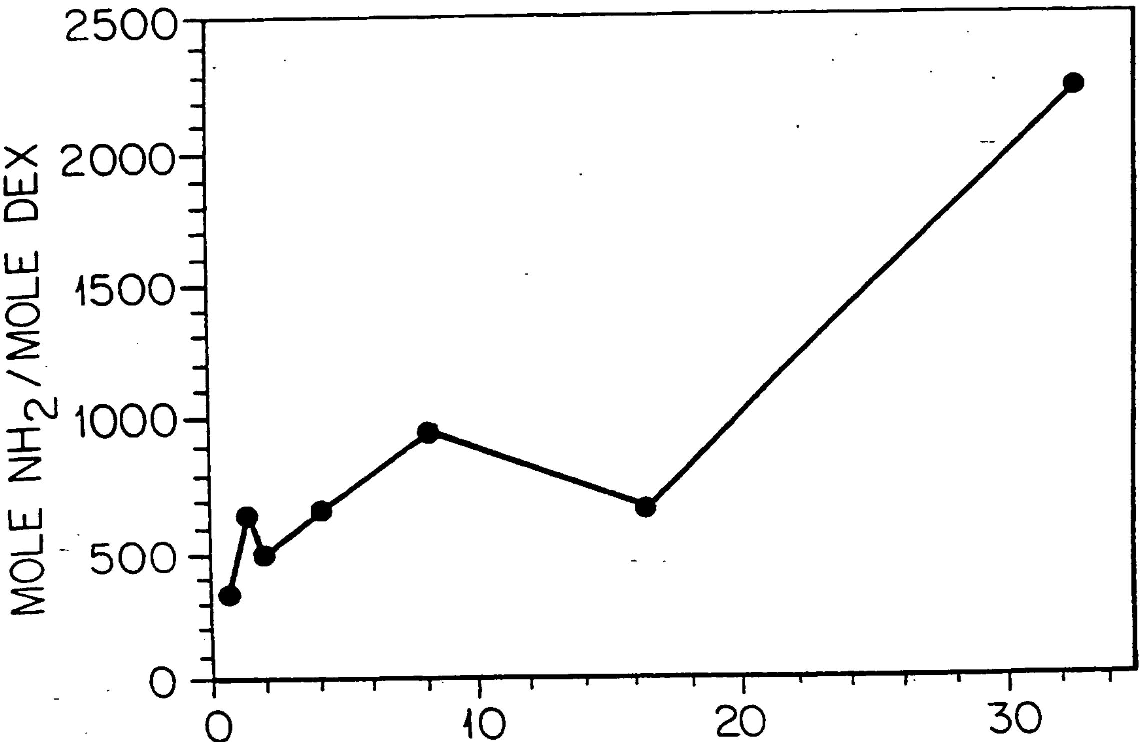

2500
2000
1500
1000
500
0
MOLE NH$_2$/MOLE DEX
0
10
20
30
MOLE CDAP/MOLE DEX (x10-3)

FIG. 4

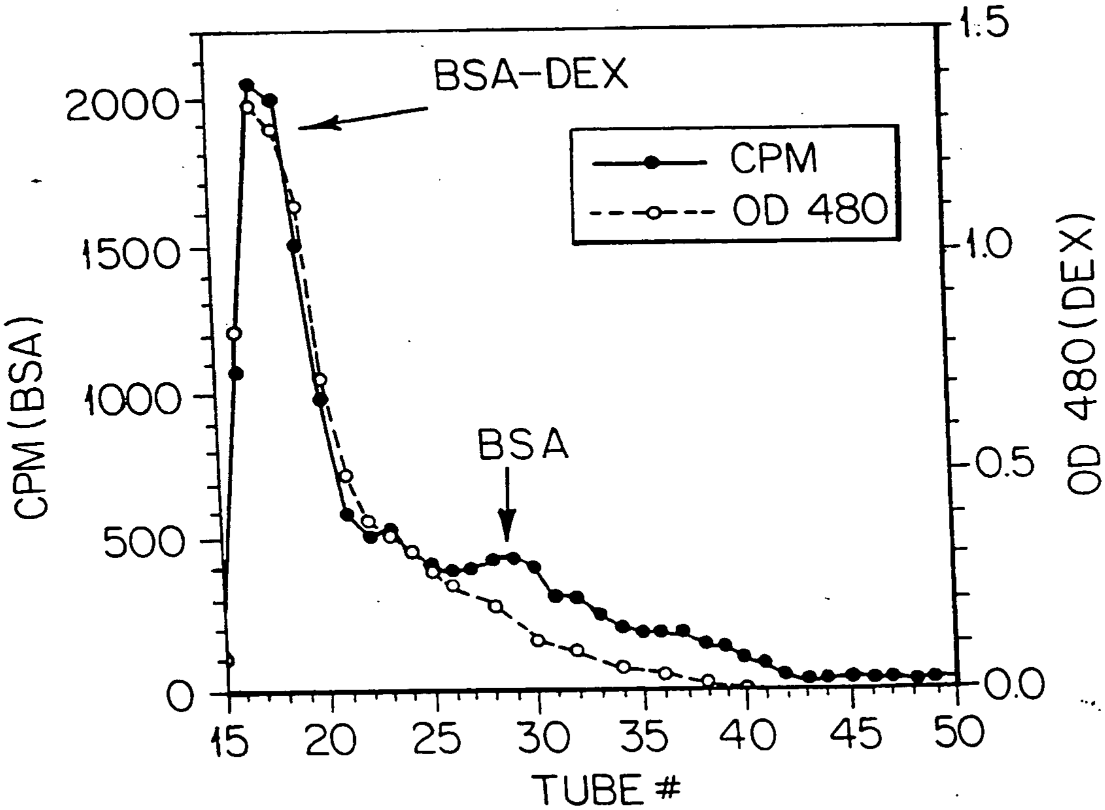
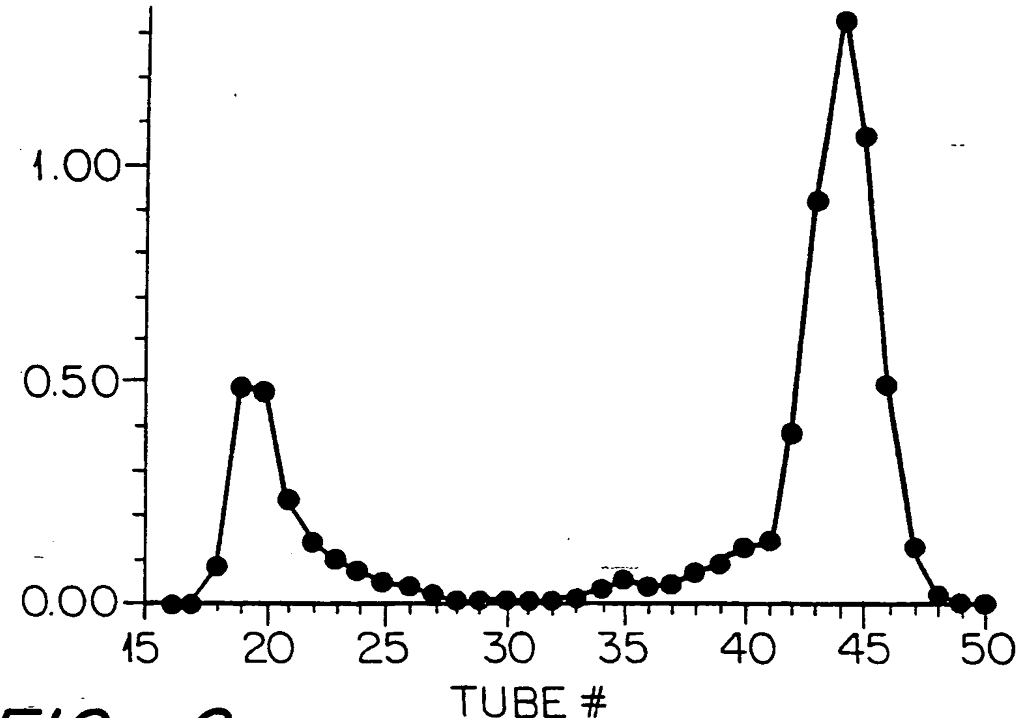
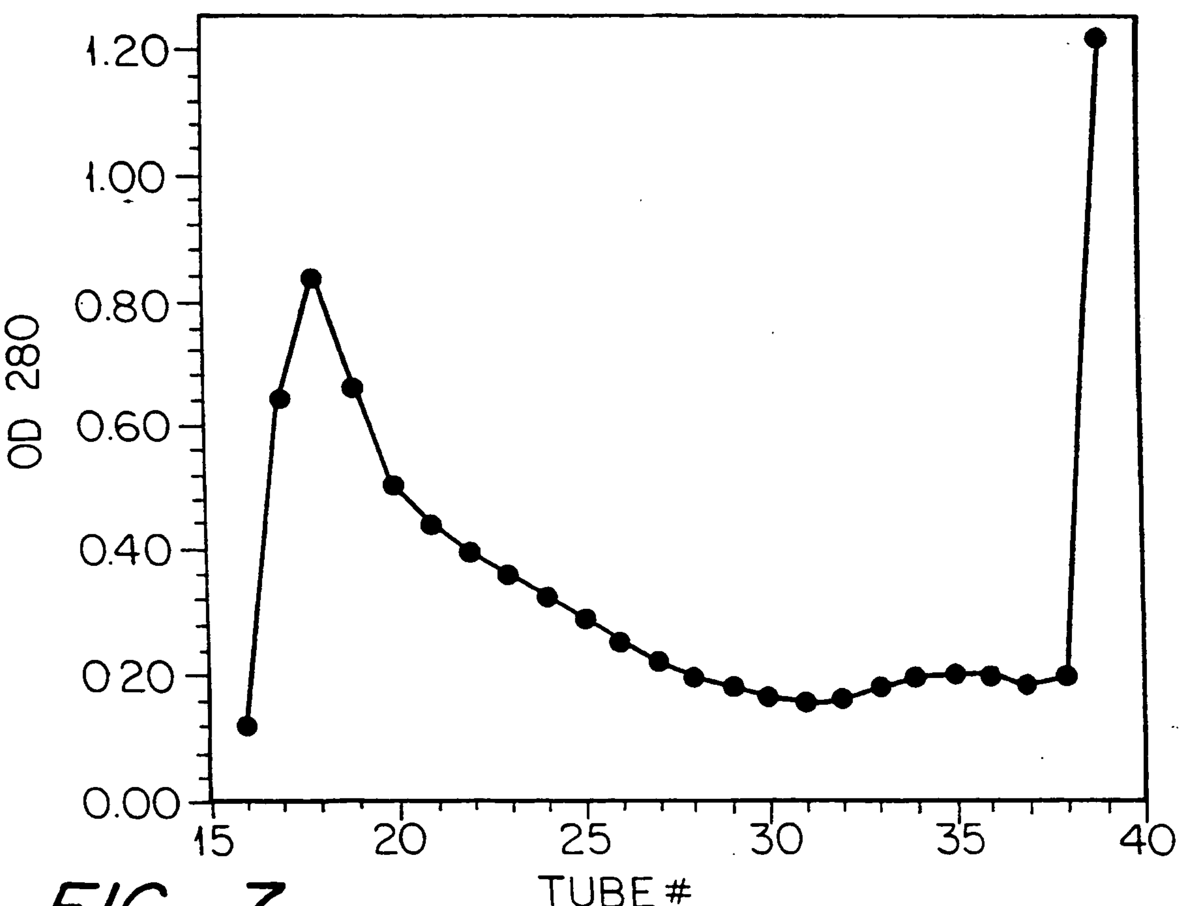

BSA-DEX
CPM
OD 480
BSA
2000
1500
1000
500
0
CPM (BSA)
1.5
1.0
0.5
0.0
OD 480 (DEX)
15
20
25
30
35
40
45
50
TUBE #

FIG. 5



1.00
0.50
0.00
OD 280
15
20
25
30
35
40
45
50
TUBE #

FIG. 6



1.20
1.00
0.80
0.60
0.40
0.20
0.00
OD 280
15
20
25
30
35
40
TUBE #

FIG. 7

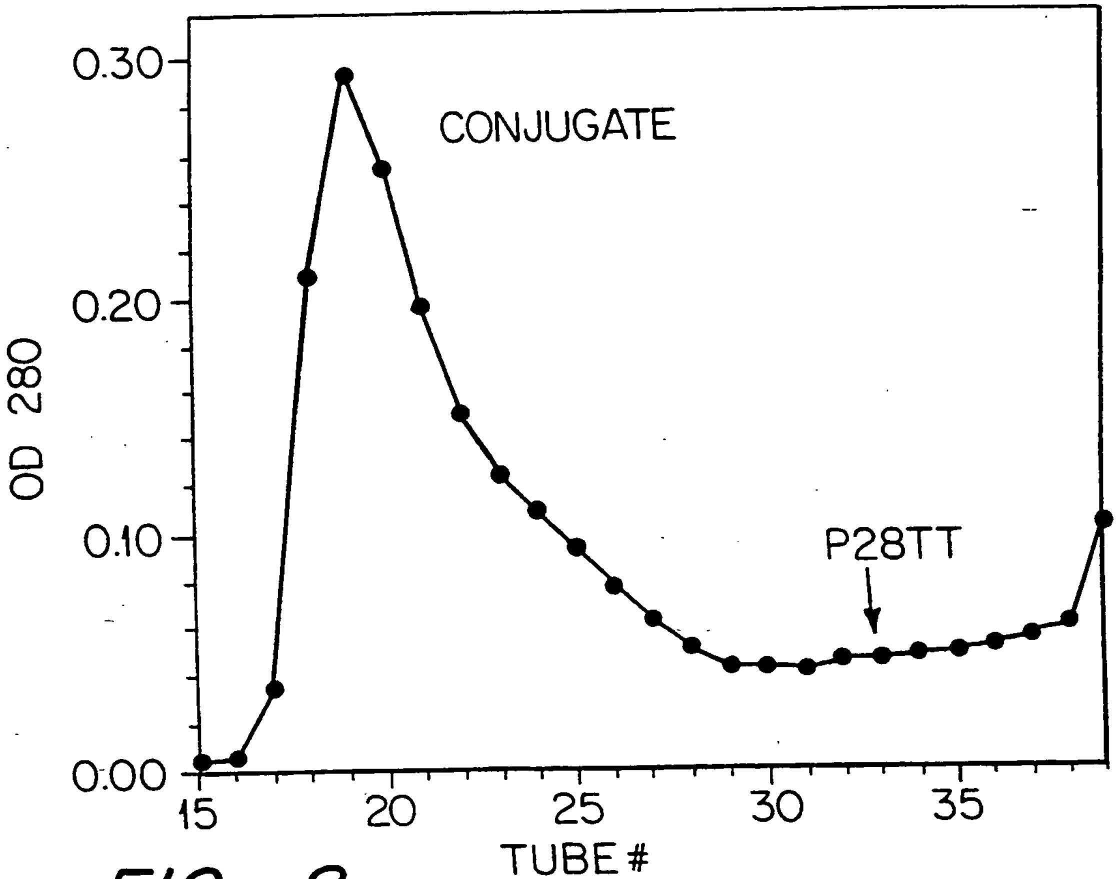

0.30
0.20
0.10
0.00
OD 280
CONJUGATE
P28TT
15
20
25
30
35
TUBE#

FIG. 8

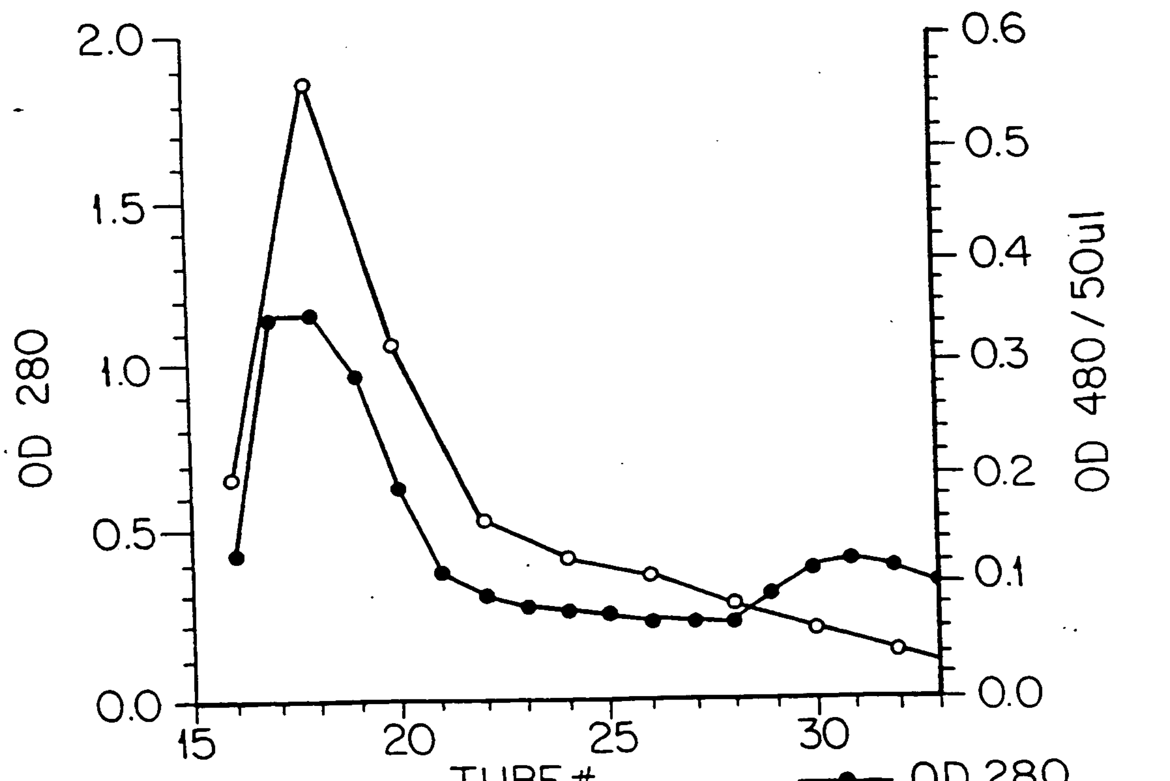

2.0
1.5
1.0
0.5
0.0
OD 280
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD 480/50ul
15
20
25
30
TUBE#
OD 280
OD 480/50ul

FIG. 9

INTERNATIONAL SEARCH REPORT

Intern   nal Application No
PCT/US 94/10658

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K39/385

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	DE,A,18 15 332 (PHARMACIA AKTIEBOLAG) 24 July 1969 see the whole document	1,3,7,8, 10 2,4-6,9, 11
Y	WO,A,93 15760 (MOND ET AL) 19 August 1993 cited in the application see page 7, paragraph 2 - page 9, paragraph 2 see page 20, paragraph 2 - page 23, paragraph 1 see page 27, paragraph 2 - page 28, paragraph 1 see page 45, paragraph 1	2,4-6,9, 11
Y	EP,A,0 186 576 (MERCK & CO.INC.) 2 July 1986 see page 8, line 3 - page 11, line 13	9
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 January 1995	10.02.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Sitch, W

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Inten nal Application No
PCT/US 94/10658

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF CHROMATOGRAPHY, vol.573, 1992, AMSTERDAM pages 132 - 135 CARPENTER ET AL 'PREPARATION OF HEPARIN-GLYCERYL CONTROLLED-PORE GLASS AFFINITY MEDIA FOR THE SEPARATION OF ALPHA- AND BETA-LIPOPROTEINS' cited in the application see the whole document ---	1-11
A	INTERNATIONAL JOURNAL OF CANCER, vol.47, no.3, 1 February 1991, GENEVA pages 439 - 444 ANDERSSON ET AL 'BINDING OF EPIDERMAL GROWTH FACTOR-DEXTRAN CONJUGATES TO CULTURED GLIOMA CELLS' cited in the application see the whole document -----	1-11

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/10658

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 10 and 11 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/10658

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
DE-A-1815332	24-07-69	BE-A-	725872	20-06-69
		CH-A-	503707	28-02-71
		FR-A-	1595332	08-06-70
		GB-A-	1247896	29-09-71
		NL-A-	6818410	24-06-69
		US-A-	3788948	29-01-74
WO-A-9315760	19-08-93	AU-B-	3722093	03-09-93
		CA-A-	2129899	12-08-93
		EP-A-	0625910	30-11-94
EP-A-0186576	02-07-86	AU-B-	584149	18-05-89
		AU-A-	5146885	26-06-86
		CA-A-	1256250	20-06-89
		DE-A-	3586398	27-08-92
		IE-B-	58977	15-12-93
		JP-A-	61186400	20-08-86
		US-A-	4830852	16-05-89